

Integrating phylogeographic and phenotypic evidence to delimit deep evolutionary lineages in the *Dryophytes japonicus* species complex, with an assessment of their conservation needs

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<https://zoobank.org/0DF57F69-F555-4428-98B1-199606FAB49E>

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Academic editor: Günter Gollmann ♦ Received 24 September 2024 ♦ Accepted 16 December 2024 ♦ Published 7 February 2025

Abstract

Proper taxonomic and phylogeographic frameworks are a prerequisite in evolutionary, ecological, and conservation research, but many species still lack adequate assessments. Earlier studies on the northeast Asian treefrog, *Dryophytes japonicus*, identified a deep Mio-Pliocene diversification; however, phenotypic assessments were lacking, and the taxonomic identity of the identified clades is under debate. In this study, we assessed the genetic, genomic, morphological, and acoustic diversity and differentiation within the *D. japonicus* complex to propose taxonomic arrangements and assess taxon-specific threats for each defined clade. Analyses of four mtDNA genes and 42.8 kb nuclear loci obtained by RAD-sequencing (RAD-seq) confirmed two distinct species-level clades that diverged ~6 Mya, one provisionally assigned to *D. japonicus* in southern Japan and mainland Asia and the other unnamed (*D. cf. japonicus*) in northern Japan and adjacent Russian islands. According to the mitochondrial data, each species is further divided into two subclades of Pleistocene age that display differences in morphological and call properties that may represent candidate subspecies: *D. j. japonicus* in southwestern Japan and *D. j. stephensi* on the Asian mainland and two unnamed lineages in Central and Northern Japan for *D. cf. japonicus*. Phenotypic differentiation between populations was only partly linked to their phylogenetic relationships. Finally, despite the relatively narrow ranges of many lineages, none crosses the threshold to be currently listed as threatened. The conservation of the diversity of the *D. japonicus* complex will necessarily require proper population monitoring and additional investigations to evaluate whether the unnamed delimited lineages merit taxonomic descriptions.

Key Words

East Palearctic, Hylidae, septentrional Asia, taxonomy, treefrog

Introduction

A first step towards addressing the current biodiversity crisis is to understand the distribution of populations, their evolutionary relationships, and how to best categorise them (Purvis and Hector 2000; Wheeler 2004; Pollock et al. 2020). Yet, defining populations and their taxonomic status remains an inherent difficulty in zoological studies (Padial et al. 2010; Pfeiler 2018). This is particularly true for species that consist of deeply-diverged clades (“species complexes”), but which have not yet received taxonomic attention due to the lack of resources, time, or interest (Britz et al. 2020). Species complexes are associated with two major challenges: the first one is determining which of the clades are genuinely threatened (Bland et al. 2015), and the second one is how to clearly define taxonomic levels (Purvis and Hector 2000). These issues are acute for amphibians (Rodrigues et al. 2010; Button and Borzée 2021) as the number of described species has been steadily increasing over the past decades (Frost 2024), while they are the most threatened vertebrate class (Stuart et al. 2004; Wake and Vredenburg 2008; Bishop et al. 2012).

Taxonomy was originally based on morphology or other phenotypic traits (Wilson and Brown 1953; Wiens and Servedio 2000), although genetic advances revealed that these are uninformative of the speciation process (Cohen 2018; Boluda et al. 2019). Consequently, species delimitation integrates genetic and phenotypic characters, generally considering mating call properties and morphology in the case of anurans (Lougheed et al. 2006; Angulo and Reichle 2008; Garda et al. 2010; Köhler et al. 2017). Some recent work further implemented current ecological requirements, habitat suitability, and niche segregation to support species differences (e.g., Dufresnes et al. 2018; Borzée et al. 2020b). All these approaches have their own limitations (Tsuji-Nishikido et al. 2012; Wang et al. 2013; Journé et al. 2020), further restricted by the fact that the concepts of “species” and “subspecies” are not agreed upon (Mallet 2001; Tobias et al. 2010; Braby et al. 2012; Stankowski and Ravinet 2021), and the way to define speciation and assign species ranks differs among taxonomists. Evolutionary biologists favour reproductive isolation to define species under the biological species concept (Stankowski and Ravinet 2021), which can be evaluated by hybridisation rates, while phylogenetics instead relies on monophyly and sequence divergence (the general lineage species concept). However, species-level divergence estimates, and especially divergence times, are inconsistent between clades

(Sturmbauer and Meyer 1992; Foote et al. 1999; Ferguson 2002), and the potential for hybridisation cannot be measured between allopatric lineages that do not coexist. To reconcile approaches, the unified species concept was proposed (de Queiroz 2005), where species are defined as monophyletic entities that show limited contemporary hybridisation. In practice, this idea can be tested through the combined analyses of phylogenetic relationships and hybrid zones in order to estimate the amount of phylogenetic divergence (Dufresnes et al. 2020b). In recent years, the field has greatly benefitted from genomic resources, e.g., “genomic phylogeographies” (Dufresnes et al. 2019b), where genome-wide multilocus data allow for correcting erroneous taxonomic decisions taken based on single-gene inferences, especially mtDNA (Wang et al. 2020; Dufresnes and Jablonski 2022). This is especially true of treefrogs from the genus *Hyla*, where the discovery and characterisation of new species by phylogenetic (Stöck et al. 2012; Dufresnes et al. 2018) and hybrid zone genomic studies (Dufresnes et al. 2020b) led to the resurrection of old nomina (e.g., *Hyla orientalis*; Bedriaga 1890 “1889”) or the description of new taxa (e.g., *Hyla perrini*; Dufresnes et al. 2018).

It is generally agreed that the taxonomy of calling anurans needs to rely on genetic/genomic evidence for evolutionary independence (Speybroeck et al. 2020), combined with call properties and morphometric analyses. In deep clades, these traits diverge in parallel, and phylogenetic patterns are generally associated with acoustic (Robillard et al. 2006; Goicoechea et al. 2010) and morphometric differences (Suwannapoom et al. 2021). However, among closely related lineages, local adaptation and phenotypic plasticity may further contribute to the variation and blur the link between call and genetic differences (Forti et al. 2017). Hence the maintenance of “cryptic” species that cannot be reliably diagnosed based on phenotypic traits.

Conservation requirements now underpin a large number of taxonomic studies (Rodrigues et al. 2010). Biodiversity is declining at an unprecedented rate (Wake 2012; Ceballos et al. 2020), and assessing each taxonomic unit separately is necessary to determine conservation units and conservation plans (daSilva et al. 2010). This is especially true of taxa with large ranges but high phylogeographic diversity, as such species complexes are generally listed as abundant despite the threats to some of their lineages, yet stable population dynamics in others, and therefore bring little conservation attention to the species as a whole. In most cases, only charismatic species receive the lineage-specific conservation attention required, and, for instance, tigers (*Panthera tigris*) are divided into

six evolutionary clades recognised as subspecies (Liu et al. 2018), with distinct conservation situations between them. Some clades increase in population size, such as in Nepal (Panthera 2018), while others are stable (Amur tiger; Miquelle et al. 2011), and some others are close to extinction (Sumatran tiger; Linkie et al. 2008) or already extinct (Caspian tiger; Jackson and Nowell 2011).

The treefrog *Dryophytes japonicus* is distributed from northern central Mongolia, across southeastern Russia, and septentrional northeast China and the Korean peninsula, as well as the Japanese archipelago except for the Ryukyu Islands (Borzée 2024). As the entire *D. japonicus* complex is currently treated as a single species by taxonomic authorities, it is currently listed as non-threatened by the IUCN Red List (ASG 2023). Phylogeographic analyses of mitochondrial and intron-based nuclear markers have revealed that this species is a species complex, with two deeply diverged clades distributed in the north-eastern Russo-Japanese islands and the western side of the Japanese Islands and mainland Asia, respectively (Li et al. 2015; Dufresnes et al. 2016). While these clades were proposed as distinct species, phenotypic differences (including bioacoustics) have not yet been assessed, and their nomenclature remains unsolved, as the vague type locality of *D. japonicus* (given as “Japan”) could apply to both (Dufresnes et al. 2016; Dufresnes and Litvinchuk 2022). In addition, both species seemingly diversified in multiple phylogeographic lineages in Japan and mainland Asia, which may also be worth considering within taxonomic and conservation frameworks. In addition, there is a strong variation in habitat use depending on regions, which calls for lineage-based threat assessments. Namely, treefrogs breed both in agricultural wetlands and lentic natural habitats in Japan, principally rely on crater lakes to breed on Jeju Island off the coast of the Korean Peninsula (Roh et al. 2014), mainly use rice paddies on the west coast of the Korean Peninsula (Borzée and Jang 2015), and predominantly inhabit oxbow lakes and other medium-sized water bodies in Mongolia and Russia (Litvinchuk et al. 2014; Borzée et al. 2019b).

In this study, we integrated multiple lines of evidence to propose taxonomic arrangements within the *D. japonicus* complex, with immediate perspectives for its conservation. We combined new mitochondrial barcoding with genomic and phenotypic analyses to (1) clarify the distribution of the identified lineages, (2) improve their delimitation in taxonomic arrangements, and (3) assign threat levels to the defined taxa and phylogeographic units.

Materials and methods

Field sampling

We collected data between 2016 and 2019 in the Republic of Korea (hereafter R Korea), the Democratic People’s Republic of Korea (hereafter DPR Korea), the People’s Republic of China (hereafter China), the Russian Federation

(hereafter Russia), Mongolia, and Japan (Fig. 1; see Suppl. material for sample size per locality and dataset). We attempted to sample the species following the range described by the Red List assessment at the time of sampling (Kuzmin et al. 2017). However, the species is not present west of the Songliao plains in northern China, with the exception of a population along the Chaogedu’er River bordering the west of the Yudaokou Grassland Forest Scenic Park at the border between Hebei and Inner Mongolia (Borzée 2024).

We aimed to obtain genetic samples, call recordings, and morphometric data for each individual collected. To do so, we first quietly waited for 5 min upon arrival at a site to locate calling males. We then recorded calling individuals with a linear PCM recorder (Tascam DR-40; California, USA) linked to a unidirectional microphone (unidirectional electret condenser microphone HT-81, HTDZ; Xi’an, China). Once recorded, the focal individual was caught and measured with a digital calliper (1108-150, Insize; USA) to the nearest 0.1 mm three times, and the measures were averaged. The continental Asian variables were randomly taken by twelve observers, while the Japanese samples were measured by three teams, with one observer overlapping with all sites to ensure continuity in the measurement methods. The clustering of observers and genetic clades do not overlap. Details for data collection are in the Suppl. material 1. Finally, each individual was orally swabbed to acquire genetic materials (cotton-tipped swab; 16H22, Medical Wire; Corsham, UK). Genetic materials were then stored at -20 °C until genetic analyses. Due to the cryptic nature of treefrogs, we did not manage to catch all the frogs recorded, and not all frogs caught could be recorded. Sample sizes are detailed below.

Sampling followed the rules and regulations of each nation where sampling was conducted. A general research visa was obtained for fieldwork in Mongolia (J22962), and fieldwork in DPR Korea was conducted under the supervision of the Ministry of Land and Environment Protection of the Democratic People’s Republic of Korea. Sampling in Japan, R Korea, China, Mongolia, and Russia did not require a research permit as the species was not threatened, and sampling was not invasive. The general study was approved by the ethics committee of Nanjing Forestry University (permit 202-20-14).

Genetic, call, and morphological analyses

For the molecular analyses, we collected tissue samples from 294 new individuals of the *D. japonicus* complex of known geographic origins, as well as one sample of the closest relative, *D. immaculatus*, as an outgroup. All analyses followed the detailed protocols found in the literature and are repeated in detail in the Suppl. material 1. Specifically, the genetic analyses relied on four partial sequences: ~900 bp of cytochrome-b (*cytb*); ~450 bp of cytochrome C oxidase subunit I (*COI*); ~1000 bp of 16S-ND1 ribosomal

large subunit gene, including adjacent tRNAs and NADH dehydrogenase subunit 1 (ND1) sequences; and ~900 bp of 12S ribosomal small subunit (12S; details in the Suppl. material 1). The subsequent analyses followed the protocol from Dufresnes et al. (2016) and included the construction of maximum-likelihood trees using PhyML 3.2 (Guindon et al. 2010), estimates of the net genetic distances between the mitochondrial clades and subclades in MEGA X (Kumar et al. 2018), and time-calibrated phylogenetic reconstruction on the concatenated mitochondrial alignment (45 haplotypes + 1 outgroup) in BEAST 2.6 (Bouckaert et al. 2019), using two calibration points. We also performed a third phylogenetic analysis with SNAPP (Leaché et al. 2014) to obtain a species tree based only on variable sites.

The call analyses generally followed the protocols from Jang et al. (2011), relying on the variable extraction from Park et al. (2013). Only individuals recorded for at least one minute were considered in the call analysis, resulting in fewer individuals ($n = 194$) recorded than genetically barcoded. We analysed the data through a principal component analysis (PCA) and assessed the variation between clades two-by-two through Tukey tests conducted in R (version 4.1.1; R Core Team 2021).

Next, for the morphological analyses, only the individuals caught after being recorded were included, resulting in fewer individuals in this analysis ($n = 314$). The analyses principally followed Borzée et al. (2013). We relied on a PCA to visualise morphological variations between populations, followed by a MANOVA to test for the presence of significant differences between clades in SPSS v.21 (SPSS, Inc., Chicago, USA).

Taxonomy

Taxonomic decisions are biased, based on a multitude of criteria, ranging from the focal taxonomic group, the definition used, and the team conducting the study. The definitions of allopatric and parapatric (sub)species are also variable. As a result, species-level taxonomic arrangements differ, with some degree of interpretation, especially for taxa that fall in the “grey zone of speciation” (Dufresnes et al. 2021). Various subspecies concepts have also been proposed (Braby et al. 2012; Hillis 2019; Hillis 2020; de Queiroz 2020). Definitions also sometimes lead to contradictory arrangements, i.e., instances of paraphyly (Jiao and Yang 2021). The biological and phylogenetic species concepts are the most generally accepted (Stankowski and Ravinet 2021), although they present some limitations in their implementations, such as not weighting phenotypic divergence as much as molecular divergence (Tobias et al. 2010). Here, we followed an integrative approach to delimit species and subspecies with respect to both genetic and phenotypic divergence found in the *D. japonicus* complex. As part of this effort, we further attempted to apply available nomina to each of the defined taxonomic units, following up on previous literature searches on this complex (Dufresnes et al. 2016).

Clade-specific threat assessment

The geographic region of this study is characterised by important environmental contrasts, including some of the largest urban centres as well as areas devoid of human presence. The developed areas also create further variations in the landscape structure due to rice agriculture close to urban environments (Lee and Miller-Rushing 2014) used as substitute habitats exploitable by the species (Naito et al. 2013; Borzée et al. 2019a). To assess threat levels, we followed the IUCN Red List categories and criteria for each lineage due to their robustness (Maes et al. 2015) and because evaluations are conducted against quantitative thresholds for five criteria (IUCN 2012): “A, population size reduction; B, geographic range size; C, small population size and decline; D, very small population and/or restricted distribution; and/or E, quantitative analysis of extinction risk.” All threats, habitats, uses, and trades are presented following the IUCN Red List criteria and categories (www.iucnredlist.org/resources/redlist-guidelines) and in the order specified.

Results

Genetic analyses

Mitochondrial barcoding of four genes (*cytb*, *COI*, 16S-ND1, 12S) for the 390 individuals confirmed that the *D. japonicus* complex is composed of two major clades: clade A in Sakhalin Island in Russia, northeastern and Central Japan; and clade B in southwestern Japan and the Asian mainland (Fig. 1). These clades come in contact in central Japan, where we barcoded both matrilineages in Kyoto. Moreover, each clade featured several lineages, respectively A1-A4 and B1-B5, which could be mapped across 66 localities. In total, we identified 135 unique haplotypes for *cytb*, 41 haplotypes for *COI*, 103 haplotypes for 16S-ND1 rRNA, and 46 haplotypes for 12S rRNA.

The four concatenated genes (3.2 kb, 45 haplotypes) provided a mostly resolved time-calibrated phylogeny of mitochondrial diversity (Fig. 1). Clades A and B diverged during the Late Miocene (6.1 Mya, 95% Highest Posterior Density (HPD): 5.3–7.0), and later diversified from the Late Pliocene onwards: the oldest intra-clade split was dated to 2.7 Mya (95% HPD: 2.1–3.0), namely between subclades A1&A2 (northern Japan) and A3&A4 (central Japan). All other splits are of Pleistocene ages, including between southern Japan (subclade B1&B2&B3) and the mainland (subclade B4&B5; 1.2 Mya, 95% HPD: 0.9–1.5). However, the phylogenetic position of the Japanese lineage B1 is not resolved, as it branches with the mainland subclades in alternative topologies.

Sequence divergences between the two major clades were high for every gene fragment (>8% at *cytb*, *COI*, and 16S-ND1 rRNA; ~3% at 12S rRNA), and relative genetic distances between lineages corresponded to their respective divergence time. In the case of lineages A3,

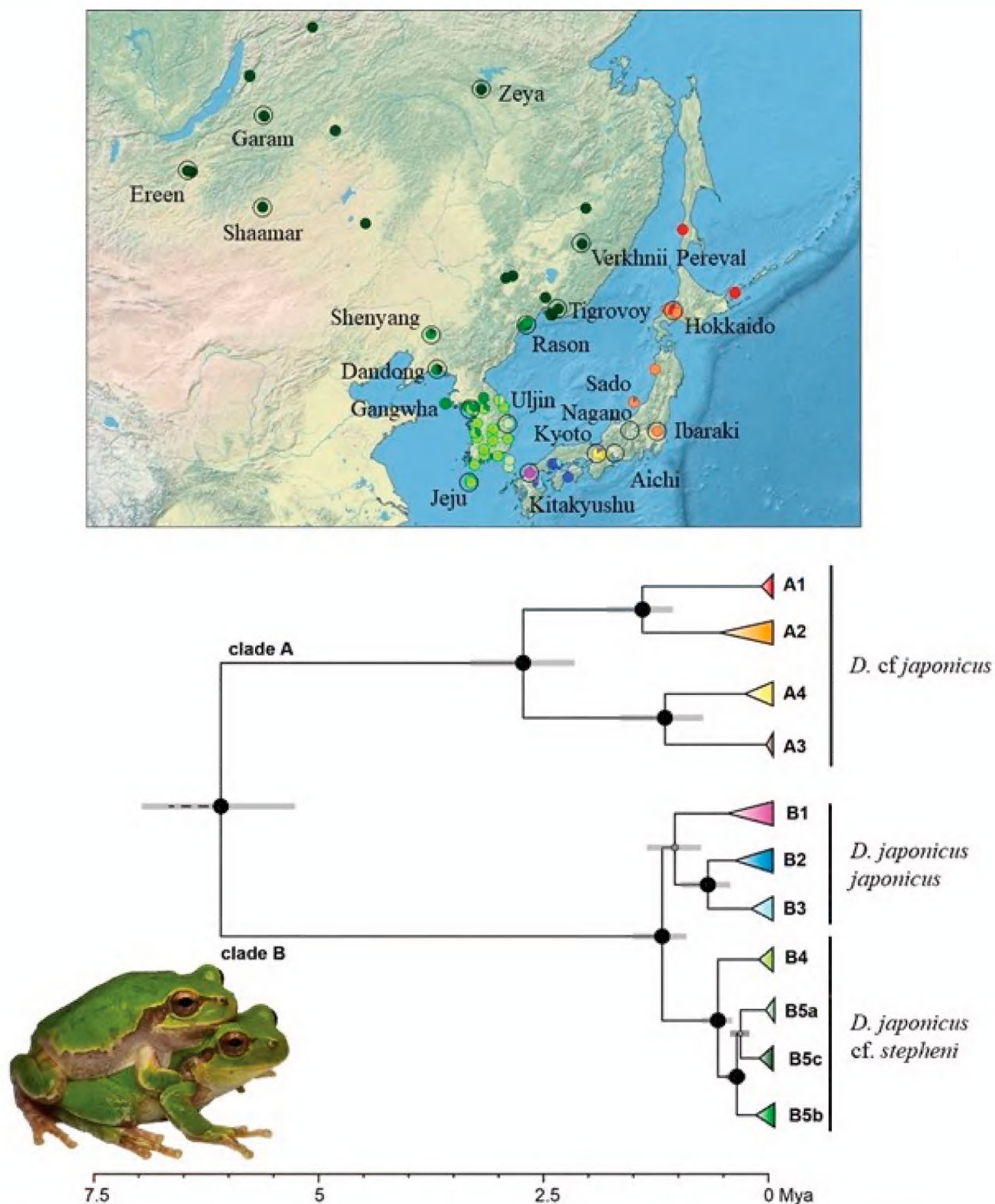


Figure 1. Sampling sites for the *Dryophytes japonicus* complex in northeast Asia. **A.** Colours represent mtDNA lineages combining data from Dufresnes et al. (2016; non-circled pie charts) and this study (labelled/encircled pie charts), which were further included in morphometric and bioacoustics analyses; **B.** Time-calibrated mitochondrial phylogeny built from concatenated sequences of four genes (3.2 kb). Node support is shown by circles with size and darkness gradients. Proposed taxonomic names are indicated. The name *ussuriensis* is available and would have priority for lineage B5 for potential taxonomic updates.

B2, and B3, only *cytb* sequences were available (Dufresnes et al. 2016), and 12S rRNA sequences could not be obtained for lineage B1 (Table 1).

The mitochondrial differentiations reflected the following geographic patterns. In clade A, lineages are arranged along a latitudinal gradient, with A1 in Sakhalin/Hokkaido (on the northern edge of the Japanese archipelago), A2 in Hokkaido/northern Honshu, A3 on Sado Island (offshore Honshu), and A4 in Central Japan (including Sado Island). In clade B, however, the three Japanese lineages (subclades B1&B2&B3) shared sympatric distributions.

Spatial partitioning was more evident for the mainland lineages: B4 was restricted to the southern parts of the Korean Peninsula and Jeju Island, while B5 was present along the Baekdudaegan mountain range on the Korean peninsula and expanded northwards into Mongolia. With our dense sampling, we could further map the distributions of three haplogroups within lineage B5: B5a in eastern mountainous Korea as well as Tsushima Island in Japan, B5b in northeastern Korea and adjacent China and Russia, and B5c in northern latitudes, including China, Russia, and Mongolia.

Table 1. Estimated divergence time (Dt, in My) and genetic distances (%) for mitochondrial and nuclear loci between the major clades and subclades of the *Dryophytes japonicus* complex. The clade A is *D. cf. japonicus*, and the clade B is *D. japonicus*.

		mitochondrial					nuclear	
		Dt	cytb	COI	16S-ND1 rRNA	12S rRNA	Dt	RAD
Clade A	clade A vs clade B	6.1	8.5	9.9	8.4	3.0%	5.9	0.23%
	A1-A2 vs A3-A4	2.7	5.1	6.4	5.3	0.7	–	–
	A1 vs A2	1.4	3.2	4.3	3.0	0.5	–	–
	A3 vs A4	1.2	3.1	–	–	–	–	–
Clade B	B1-B3 vs B4-B5	1.2	2.0	2.4	1.6	–	–	–
	B1 vs B2-B3	1	2.3	–	–	–	–	–
	B2 vs B3	0.7	1.5	–	–	–	–	–
	B4 vs B5	0.6	1.2	0.9	1.0	0.1	–	–

The *Hyla/Dryophytes* nuclear sequence alignment (43.8 kb containing 2,302 SNPs) provided identical topologies for the three methods of phylogenetic reconstruction—PhyML and BEAST on concatenated alignments and SNAPP on variable sites (Fig. 2). All internal nodes were fully supported, and the trees recovered strong nuclear divergence between *D. japonicus* samples from clade A (represented by A2 samples) and clade B (represented by B2 samples). Their estimated age was 5.9 Mya (95% HPD: 3.5–9.9), which is very close to the mitochondrial estimates. The net genetic distance between the two nuclear clades was 0.23% in our RAD-seq concatenated alignment.

Call properties

The 194 individuals recorded were grouped into the four identified subclades, with sample sizes as follows: A1&A2: $n = 41$ (831 notes), A3&A4: $n = 33$ (717 notes), B1&B2&B3: $n = 14$ (273 notes), B4&B5: $n = 120$ (2329 notes). The PCA to identify the independent dimensions of the call properties between these four groups resulted in three principal components (PCs), with eigenvalues ranging from 1.08 to 1.49, explaining a cumulated 74.09% of the variance (Table 2). A variable was judged to be important if displaying a loading factor > 0.69 , so that each variable loaded into one of the PCs. Based on the variables loading onto each of the PCs, we assigned PC1 to high and dominant frequencies, PC2 to note duration and effective amplitude, and PC3 to fundamental frequency (Table 2).

The MANOVA with the four subclades as independent variables was significant for all PCs when using individual identification numbers as a random variable (Table 2). However, the results of the Tukey tests were less uniform, especially for subclades B1&B2&B3 (Table 3). B1&B2&B3 was not significantly different from A3&A4 for PC1 and from A1&A2 and B4&B5 for PC2. In addition, A1&A2 and B4&B5 were not significantly different for PC2. It is interesting to note that this absence of significant difference is generally consistent for groups isolated by sea straits.

Table 2. Principal components and their characteristics resulting from the PCA were computed to segregate acoustic traits between the *Dryophytes japonicus* clades. In bold are the variables loading the most into each of the PCs and significant p-values from the statistical analysis to test for differences between clades.

	PC1	PC2	PC3
Note duration (s)	-0.242	-0.695	-0.184
Fundamental frequency (Hz)	-0.025	-0.028	0.932
High frequency (Hz)	0.869	-0.065	-0.238
Dominant frequency (Hz)	0.731	0.189	0.398
Call amplitude (RMS)	-0.145	0.816	-0.173
Eigenvalue	1.49	1.13	1.08
Percentage variance	29.9	22.51	21.68
MANOVA			
χ^2	112.44	46.83	161.90
Df, Df error	3, 4146	3, 4146	3, 4146
F	181.42	15.85	187.44
p	<0.001	<0.001	<0.001

Table 3. Two-by-two Tukey tests to segregate acoustic traits between the *Dryophytes japonicus* clades in northeast Asia followed the phylogenetic segregation. In bold are p-values showing a significant difference between clades. Note that the clade B1&B2&B3 had the lowest number of significant two-by-two Tukey tests for all three PCs. The clade A is *D. cf. japonicus*, and the clade B is *D. japonicus*.

Variable	Clade 1	Clade 2	Mean difference (1-2)	SE	p
PC1	A1&A2	A3&A4	-0.19	0.04	<0.001
PC1	A1&A2	B1&B2&B3	-0.17	0.06	0.014
PC1	A1&A2	B4&B5	1.13	0.03	<0.001
PC1	A3&A4	B1&B2&B3	0.02	0.06	0.986
PC1	A3&A4	B4&B5	1.31	0.03	<0.001
PC1	B1&B2&B3	B4&B5	1.29	0.05	<0.001
PC2	A1&A2	A3&A4	-0.29	0.05	<0.001
PC2	A1&A2	B1&B2&B3	-0.06	0.07	0.833
PC2	A1&A2	B4&B5	0.06	0.04	0.484
PC2	A3&A4	B1&B2&B3	0.23	0.07	0.006
PC2	A3&A4	B4&B5	0.34	0.04	<0.001
PC2	B1&B2&B3	B4&B5	0.12	0.06	0.264
PC3	A1&A2	A3&A4	-0.21	0.05	<0.001
PC3	A1&A2	B1&B2&B3	-0.67	0.07	<0.001
PC3	A1&A2	B4&B5	0.41	0.04	<0.001
PC3	A3&A4	B1&B2&B3	-0.46	0.07	<0.001
PC3	A3&A4	B4&B5	0.62	0.04	<0.001
PC3	B1&B2&B3	B4&B5	1.08	0.06	<0.001

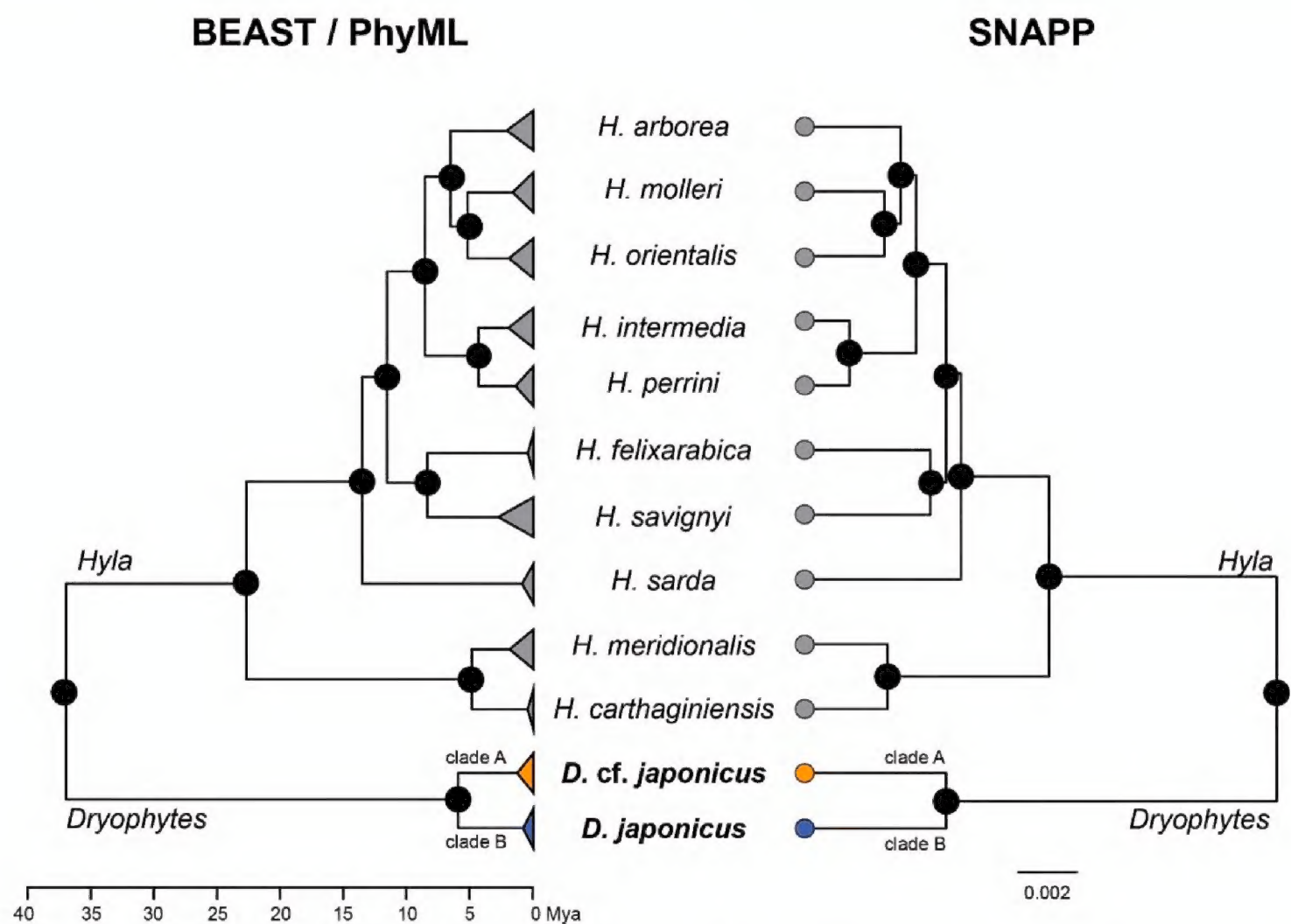


Figure 2. Phylogenetic reconstructions of nuclear sequences obtained by RAD-seq (generated by Dufresnes et al. 2018), representative of European hylids and the two major clades of the *Dryophytes japonicus* complex. The left phylogeny was obtained by reconstruction methods based on concatenated alignments (variable and invariable sites), namely BEAST and PhyML. Both provided identical results, and only the BEAST (time-calibrated) tree is shown. The right phylogeny was built only from variable sites (SNPs) with SNAPP, also showing an identical species tree. All nodes were fully resolved (large dark circles) in all three analyses.

A general pattern of differentiation was apparent when plotting all three PCs together in a three-dimensional space (Suppl. material 1). In particular, subclade B4&B5 (Asian mainland) formed a distinct cluster, which is also seen from the estimates (Table 4), especially ND and call amplitude (higher for B4&B5) and frequency-related measurements (lower for B4&B5). The values for A1&A2 were similar, except for the dominant frequency and call amplitude (Table 4). We do not expect HW to have an impact of variations in call properties among clades as the variables overlap for all clades (see results). The values for B1&B2&B3 were generally intermediates to those of other groups.

Morphometrics

The adjusted morphometric measurements showed a greater divergence for B4&B5, the mainland Asian clade, compared to the others (Table 5). However, the directionality of the divergence compared to the other clades was not always consistent, and, for instance, the clade showed the highest and lowest values for the adjusted HLL, while it had the highest MTW and lowest HW (Table 5).

The PCA used to discriminate the morphological variations between subclades yielded four significant PCs (Table 6), explaining 62.13% of the variance. PC1 was related to head characteristics, PC2 to limb morphology, and PC3 and PC4 were restricted to single characters:

Table 4. Call properties for all subclades of the *Dryophytes japonicus* complex in northeast Asia. These values are after correction for temperature variation (20.4 °C). The clade A is *D. cf. japonicus*, and the clade B is *D. japonicus*.

		Mean	SD	Minimum	Maximum
Note duration	A1&A2	0.12	0.02	0.08	0.15
	A3&A4	0.11	0.02	0.05	0.17
	B1&B2&B3	0.11	0.01	0.08	0.18
	B4&B5	0.13	0.03	0.07	0.30
Fundamental frequency	A1&A2	754.80	162.69	190.47	1349.24
	A3&A4	744.97	201.55	290.21	1450.71
	B1&B2&B3	780.93	197.56	357.15	1246.15
	B4&B5	634.74	181.04	125.63	1391.52
High frequency	A1&A2	26254.81	9223.37	11081.10	44520.50
	A3&A4	24252.78	9223.37	9400.30	41443.70
	B1&B2&B3	20186.72	5131.74	8622.10	24521.70
	B4&B5	14133.24	5077.92	2638.34	28202.98
Dominant frequency	A1&A2	2871.25	437.73	1179.93	3646.50
	A3&A4	3142.37	563.50	1367.84	3779.54
	B1&B2&B3	3345.41	126.77	2064.77	3701.27
	B4&B5	2656.54	294.65	169.87	3331.86
call amplitude (RMS)	A1&A2	14144.04	9223.37	-1846.40	35080.90
	A3&A4	22673.66	3141.18	20251.50	33227.10
	B1&B2&B3	-15773.29	2395.56	-18629.70	-9981.90
	B4&B5	34294.88	9223.37	-73390.20	1090918.52

the distance between eyes and nostrils (EN) and the head length (HLt), respectively. All scores among subclades were significantly different (Table 6). However, only a few pairs of subclades significantly differed according to the pairwise post hoc Tukey tests (Table 7). The subclades

Table 5. Morphological data for the *Dryophytes japonicus* clades in northeast Asia. These values are after correction for snout-vent length, and abbreviations are explained in the text. The clade A is *D. cf. japonicus*, and the clade B is *D. japonicus*.

Variable	Clade	Mean	SD	Minimum	Maximum
Adjusted HLL	A1&A2	1.44	0.08	1.26	1.59
	A3&A4	1.39	0.13	1.12	1.54
	B1&B2&B3	1.56	0.12	1.40	1.76
	B4&B5	1.69	0.46	0.90	3.23
Adjusted MTW	A1&A2	0.10	0.02	0.06	0.20
	A3&A4	0.11	0.03	0.05	0.16
	B1&B2&B3	0.11	0.01	0.10	0.14
	B4&B5	0.14	0.04	0.04	0.27
Adjusted IND	A1&A2	0.08	0.01	0.06	0.12
	A3&A4	0.08	0.01	0.05	0.10
	B1&B2&B3	0.07	0.01	0.06	0.08
	B4&B5	0.08	0.01	0.04	0.12
Adjusted HW	A1&A2	0.36	0.03	0.31	0.41
	A3&A4	0.38	0.02	0.34	0.43
	B1&B2&B3	0.38	0.02	0.35	0.40
	B4&B5	0.34	0.03	0.13	0.52
Adjusted EL	A1&A2	0.11	0.01	0.09	0.15
	A3&A4	0.11	0.01	0.09	0.13
	B1&B2&B3	0.12	0.00	0.11	0.12
	B4&B5	0.11	0.02	0.05	0.26
Adjusted EAD	A1&A2	0.18	0.02	0.14	0.21
	A3&A4	0.20	0.02	0.17	0.23
	B1&B2&B3	0.20	0.01	0.19	0.23
	B4&B5	0.19	0.02	0.08	0.29
Adjusted EPD	A1&A2	0.28	0.02	0.21	0.33
	A3&A4	0.31	0.01	0.28	0.34
	B1&B2&B3	0.31	0.01	0.29	0.33
	B4&B5	0.30	0.03	0.17	0.52
Adjusted EN	A1&A2	0.09	0.01	0.07	0.12
	A3&A4	0.08	0.01	0.05	0.10
	B1&B2&B3	0.09	0.00	0.08	0.09
	B4&B5	0.08	0.02	0.04	0.37
Adjusted NL	A1&A2	0.09	0.01	0.07	0.10
	A3&A4	0.08	0.01	0.06	0.10
	B1&B2&B3	0.08	0.01	0.07	0.09
	B4&B5	0.08	0.01	0.03	0.13
Adjusted HLt	A1&A2	0.30	0.03	0.24	0.37
	A3&A4	0.28	0.01	0.26	0.31
	B1&B2&B3	0.29	0.02	0.25	0.32
	B4&B5	0.28	0.03	0.14	0.40
Adjusted TD	A1&A2	0.06	0.01	0.04	0.08
	A3&A4	0.06	0.01	0.04	0.09
	B1&B2&B3	0.07	0.01	0.05	0.07
	B4&B5	0.06	0.01	0.03	0.10

B1&B2&B3 did not significantly differ from any other subclades for any of the PCs, but all other subclades differed from the others for two to three PCs. In addition, the centroids were differentiated for all subclades when plotting the PCs against each other two by two (Fig. 3). When looking at each morphological variable independently, most averages were different from each other despite overlaps in values (Table 5, Fig. 4). The subclade B4&B5 generally had longer limbs than the others, but the head was generally longer for A1&A2 and broader for A3&A4 and B1&B2&B3.

Table 6. Principal components and their characteristics resulting from the PCA were computed to segregate morphological traits between the *Dryophytes japonicus* clades. In bold are the variables retained as loading into one of the PCs and significant p-values from the statistical analysis to test for differences between clades.

	PC1	PC2	PC3	PC4
HLL	0.28	-0.52	-0.44	-0.03
MTW	0.27	0.66	-0.19	0.14
IND	0.53	-0.13	0.02	-0.22
HW	0.61	-0.40	0.21	-0.29
EL	0.62	0.22	-0.30	0.30
EAD	0.58	0.37	0.23	-0.51
EPD	0.76	0.24	-0.13	-0.25
EN	0.34	0.22	0.59	0.18
NL	0.56	-0.62	0.02	0.12
HLt	0.43	-0.13	0.50	0.56
TD	0.58	0.13	-0.40	0.32
Eigenvalue	3.06	1.58	1.18	1.02
Percentage variance	27.78	14.35	10.74	9.26
ANOVA				
χ ²	4.66	10.78	11.28	12.47
Df, Df error	3.313	3.313	3.313	3.313
F	4.83	11.9	12.53	14.03
p	0.003	< 0.001	< 0.001	< 0.001

Taxonomic considerations

The two main clades, A (Russian and Japanese islands) and B (southern Japan and Asian mainland), that form the *D. japonicus* complex were previously considered independent species given their deep mitochondrial divergence and private alleles at intron markers, as initially proposed by Dufresnes et al. (2016) and recently reviewed (Dufresnes and Litvinchuk 2022). We hereby follow this arrangement, which is further supported by our new phylogenomic analyses that unambiguously confirm their authenticity (Fig. 1). Each species consists of two subclades, which we hereby delimit as subspecies, with respect to mitochondrial divergence (Fig. 1), as well as morphological and acoustic uniqueness (Fig. 3; Suppl. material 1). Moreover, each subclade/subspecies features various mitochondrial lineages that will warrant further phenotypic and taxonomic investigations.

The main issue in naming the species and subspecies that compose the *D. japonicus* complex relates to which one the nomen *japonicus* should apply. As reviewed by Dufresnes et al. (2016), the oldest taxon corresponding to the complex is *Hyla arborea* var. *japonica* Günther, 1858. It was described from three syntypes deposited in the British Museum of Natural History (BMNH 44.2.22.107), but originally transferred from the Leiden (“Leyden”) collection. These specimens were collected by Siebold and Bürger (Temminck and Schlegel 1838) under the entry “RMNH.RENA.1701, *Hyla arborea*, 17 ex., collected by: Siebold & Bürger, locality: Japan.” The type locality of *japonica* thus corresponds to “Japan,” but because the country is inhabited by both clades A and B,

Table 7. Two-by-two Tukey tests to segregate morphological traits between the *Dryophytes japonicus* clades in northeast Asia. In bold are p-values showing a significant difference between clades. The clade A is *D. cf. japonicus*, and the clade B is *D. japonicus*.

Variable	Region 1	Region2	Mean difference (1-2)	SE	<i>p</i>
PC1	A1&A2	A3&A4	0.19	0.23	0.844
	A1&A2	B1&B2&B3	0.36	0.35	0.730
	A1&A2	B4&B5	0.57	0.16	0.003
	A3&A4	B1&B2&B3	0.16	0.36	0.968
	A3&A4	B4&B5	0.38	0.19	0.197
	B1&B2&B3	B4&B5	0.21	0.32	0.907
PC2	A1&A2	A3&A4	-1.31	0.23	< 0.001
	A1&A2	B1&B2&B3	-0.78	0.33	0.092
	A1&A2	B4&B5	-0.40	0.16	0.057
	A3&A4	B1&B2&B3	0.53	0.35	0.427
	A3&A4	B4&B5	0.91	0.18	< 0.001
	B1&B2&B3	B4&B5	0.38	0.31	0.606
PC3	A1&A2	A3&A4	-0.10	0.23	0.973
	A1&A2	B1&B2&B3	-0.60	0.33	0.276
	A1&A2	B4&B5	-0.82	0.16	< 0.001
	A3&A4	B1&B2&B3	-0.50	0.35	0.469
	A3&A4	B4&B5	-0.73	0.18	< 0.001
	B1&B2&B3	B4&B5	-0.22	0.31	0.886
PC4	A1&A2	A3&A4	0.87	0.23	< 0.001
	A1&A2	B1&B2&B3	0.67	0.33	0.183
	A1&A2	B4&B5	1.02	0.16	< 0.001
	A3&A4	B1&B2&B3	-0.20	0.34	0.939
	A3&A4	B4&B5	0.15	0.18	0.841
	B1&B2&B3	B4&B5	0.35	0.30	0.658

more accurate details of the syntypes’ origin are necessary to decisively apply this name to one species. In the absence of direct evidence (syntype barcoding), here we rely on two anecdotal clues that suggest that the type locality of *H. japonica* is located in southern rather than northern Japan and would thus more preferentially apply to the southern clade (B). First, Dufresnes et al. (2016) noted that Siebold was posted in Nagasaki prefecture,

namely Dejima Island off Kyushu, and although he did eventually travel to eastern Japan (Tokyo area, inhabited by clade A), the Nagasaki region was the only area easily accessible to westerners in mid-19th century Japan. Accordingly, similar conclusions were reached to assign available names that were likewise described from “Japan” for other newly delimited anuran species (e.g., *Glandirana rugosa*, *Rana japonica*, *Zhangixalus schlegelii*; Dufresnes and Litvinchuk 2022). Second, we note here that Boulenger, a contemporary and acquaintance of Günther, differentiated *Hyla stepheni* Boulenger 1888 “1887,” inhabiting Geomun Island (also named Geomundo, where “do” means island in Korean, and “Port Hamilton” from a colonial perspective) in Korea, from the Japanese *H. arborea* var. *japonica*, specifically referring to Kyushu in his distinction (Boulenger 1882; Boulenger 1888 “1887”). Given the current knowledge, we assign the nomen *D. japonicus* to the southern Japanese and Asian mainland species (clade B). As a common English name, we recommend using “Northern Asian treefrog” for this species.

In the Northern Asian treefrog *D. japonicus*, the Japanese (subclade B1&B2&B3) and mainland (subclade B4&B5) populations are distinguished by mitochondrial differentiation dated to the Mid-Pleistocene (Fig. 1), as well as specific morphological and call properties (Fig. 3; Suppl. material 1). We thus propose to provisionally consider them as subspecies, with the Japanese populations (B1&B2&B3) corresponding to *Dryophytes japonicus japonicus*. We note that *Hyla heinzsteinitzi* Grach, Plessner & Werner, 2007, described from Israel (type locality: “Mamilla reservoir, IG 1710 1317, Jerusalem, Israel”) is a junior synonym. Soon after its description, genetic barcoding revealed that this species corresponded to introduced populations of *D. japonicus* in the outskirts of Jerusalem (Stöck et al. 2008) and was accordingly synonymized (Duellman et al. 2016). Specifically, the *cytb* haplotype of *heinzsteinitzi* (FJ226937) branches with

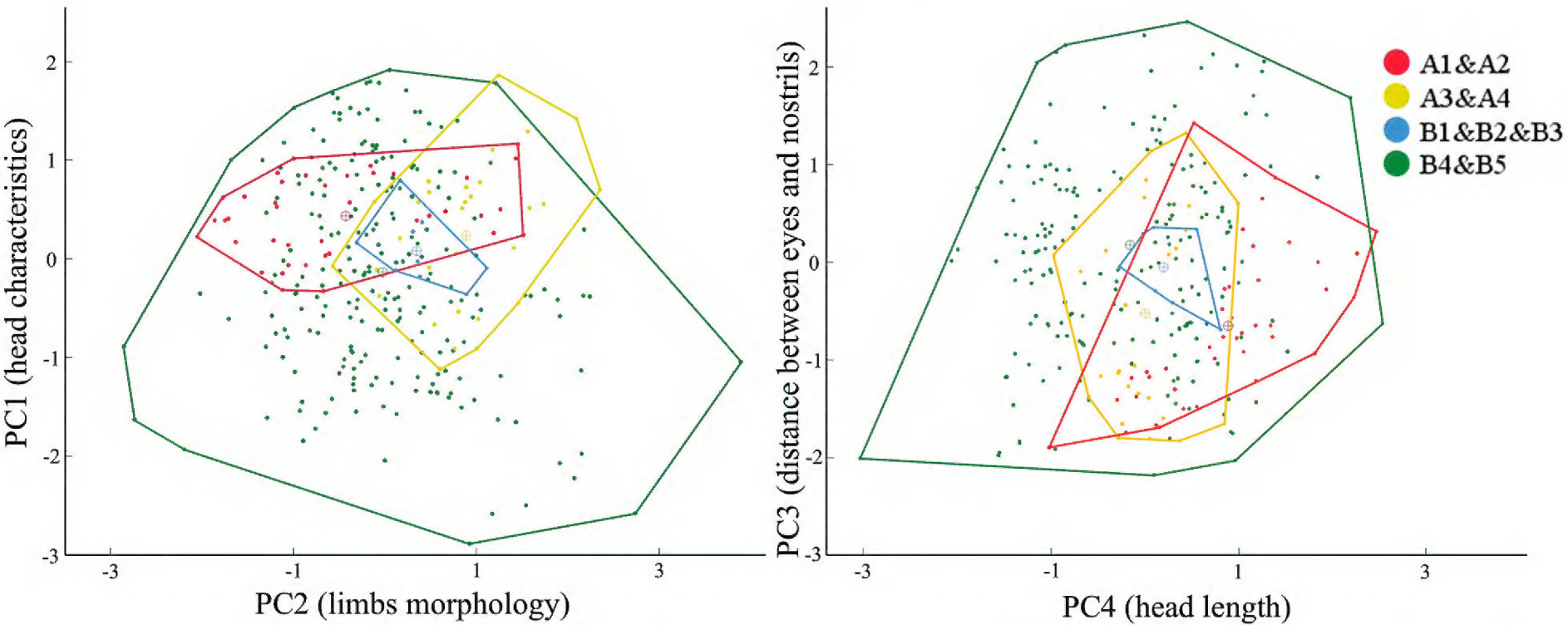


Figure 3. Scatter plots of individual morphometrics, based on the significant variables Principal Components highlighted PCA. Colours discriminate subclades.

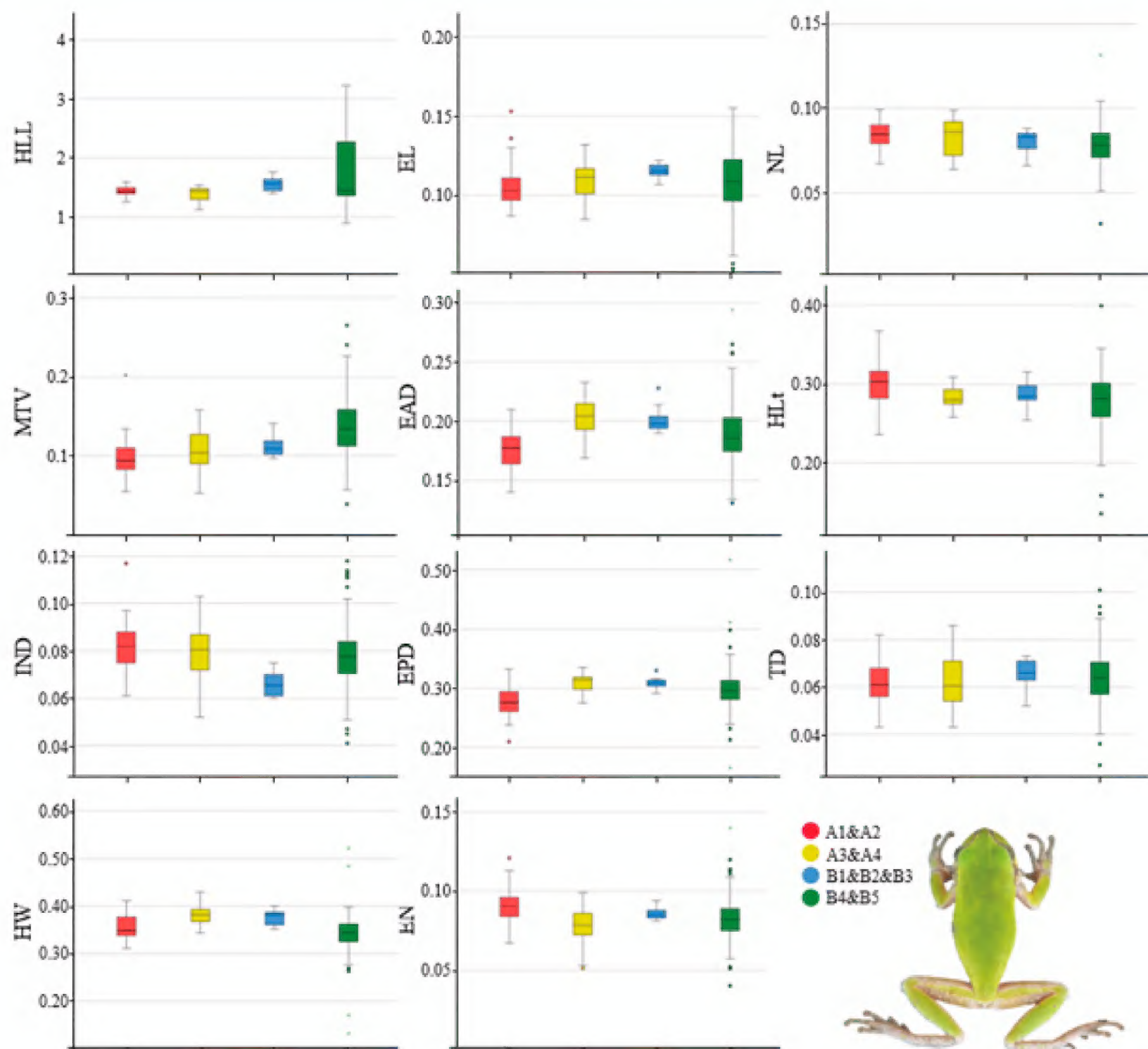


Figure 4. Comparisons of each morphological character (adjusted by SVL, thus the data is dimensionless) among the four delimited subclades. We measured 12 characters known to be informative of *Dryophytes* sp. morphology, and abbreviations are as follows. SVL: snout-vent length; HLL: hind-limb length; MTV: toe webbing length between 2nd and 3rd toes; IND: inter-nostril distance; HW: head width; EL: eye length; EAD: distance between the anterior corners of the eyes; EPD: distance between the posterior corners of the eyes; EN: eye to nostril; NL: nostril to lip; HLT: head length; and TD: tympanum diameter. Each variable was measured three times and averaged. The boxplots show medians, quartiles, and outliers.

our Japanese lineage B3. The name would be available to rename the Japanese subclade B1&B2&B3 if future research were to show that the nomen *japonicus* in fact belongs to the northern species (clade A).

Several names are available for the mainland lineages (B4&B5). The oldest one appears to be *Hyla stepheni* Boulenger 1888 “1887,” which was first synonymised with *H. japonica* by Pope and Boring (1940) and confirmed by Liu and Hu (1961). The holotype of *H. stepheni* (now BMNH 1947.2.30.99) originates from Geomun Island, as considered by several sources (Stejneger 1907; Nikolskii 1918; Condit 1964; Shannon 1956; Park et al. 2014), which is inhabited by lineage B4. The holotype is, however, unfit for new morphological measurements as it was already “dried up” in 1898 (Stejneger 1907; Shannon

1956). The identification key from Stejneger (1907) for *H. stepheni*, based on specimens from the Ussuri basin (Russia), states that “digital disks much smaller than inner metatarsal tubercles” and that the venter is different from that of specimens seen on the Japanese mainland (Hondo). However, Shannon (1956) checked Korean samples and concluded that the relative size of the inner metatarsal tubercle was not useful to identify *H. stepheni* from *H. japonica* and rejected the specific validity of *Hyla stepheni*. Since no detailed morphological comparison has been conducted after Shannon (1956), and our PCA analysis failed to detect any significant differences between B1&B2&B3 and B4&B5 (Table 7), we cannot ascertain whether the genetic gaps found in mtDNA match with discrete morphological traits. Here, we preliminarily call the

Asian mainland populations (B4&B5) *Dryophytes japonicus* cf. *stepheni*, with Geomun's treefrog as a common English name, and the willingness to avoid any colonial heritage with the name Stephen's treefrog. More detailed morphological comparisons or other genetic tools (i.e., SNPs) should be conducted to evaluate this subspecies.

Other names that also correspond to the mainland subspecies of *D. japonicus* are available (Kuramoto 1980). First, *Hyla sodei-campi* Kostin, 1935, was described from China, with the type locality given as “Mankou of the Western line of North Manchurian Railway (Chinese Eastern Rly.) in Soda Steppe, N. Manchuria” (Kostin 1935). This taxon was omitted in recent phylogeographic and taxonomic work due to its synonymy with *D. japonicus* (Duellman et al. 2016; Dufresnes et al. 2016). Second, *Hyla arborea ussuriensis* Nikolskii 1918 was described from Russia with the type locality restricted to “Chernigovka railway station, Primorskaya oblast” (Vedmederya et al. 2009). Both correspond to lineage B5 (Fig. 1), so we provisionally treat them as synonyms of *D. j. stepheni*, noting that the names are available for further taxonomic distinctions between and within subclades B4 and B5.

To the best of our knowledge (see also Frost 2024), no taxon corresponding to the northern candidate species (clade A) has been described, so this species remains nameless. We thus labelled it here as *D. cf. japonicus*. Given their significant mitochondrial and phenotypic differences, we also propose to distinguish populations from southern Honshu (A3&A4) and northern Honshu / Hokkaido / Sakhalin (A1&A2). These lineages are candidates for subspecies descriptions, provided that their divergences are not limited to mtDNA but are also confirmed by multilocus nuclear data.

Threat assessments

The four lineages delimited as candidate species or subspecies in the *D. japonicus* complex occur in areas where landscapes are degraded by residential and commercial developments, but they are not currently used or traded for human consumption. They were, however, used as food complements, especially live young metamorphs, during the last century when war conditions made food accessibility unreliable in Korea. At the macroscale, the habitat used by the two species is similar. Both inhabit mixed and deciduous broadleaved forests, planted forests, forest steppes, bushlands, meadows, swamps, paddy fields, small ponds, slow streams, and the surrounding vegetation—although clade A is also present in planted forests of *Cryptomeria* in Japan. At a smaller scale, the different subclades were present in different habitats due to human changes to the landscape. Lineages found at the southern latitudes (B1&B2&B3, A3&A4) occur in large numbers in agricultural wetlands and are also present in natural landscapes. This substitute habitat is, however, increasingly mechanised and replaced by human infrastructures related to the manufacture of goods, which do not allow

amphibian species to thrive (Fujioka and Lane 1997; Park et al. 2021). The northern lineages (B5 and A1) are principally found in natural landscapes, and especially in the vegetation surrounding small and oxbow lakes (Litvinchuk et al. 2014; Borzée et al. 2019b), but they are also impacted by landscape changes (Borzée et al. 2021a). All clades do, however, occur in protected areas (<https://www.protectedplanet.net/>). Finally, none of the lineages is known to be significantly impacted by invasive species, although they are sometimes preyed on by the introduced *Lithobates catesbeianus* (Groffen et al. 2019). Similarly, batrachochytrids are not known to be lethally impacting the species complex (Borzée et al. 2017).

Climate change is expected to negatively affect the lineage studied here. A conservative prediction based on the Paris Climate Agreements (COP21 2015) determined that climate change will result in a moderate-to-high-risk (IPCC 2014; IPCC 2018). Namely, under the 1.5 °C scenario, the air temperature will increase by 2.7 °C and 3.3% humidity in northeast Asia (peaking at 7.0 °C and 13% if the Paris Climate Agreements are not held; Xu et al. 2017).

Subclade A1&A2: northern subspecies of *D. cf. japonicus*

While the population size of this lineage has not been quantified, the threats highlighted above might cause local declines, as defined by the IUCN Red List categories and criteria (IUCN 2012). The population size is, however, likely to be higher than 10,000 individuals (criterion C), and the decline is unlikely to be large enough ($\geq 30\%$; criterion A) for the clade to be listed as threatened. Similarly, while the extent of occurrence and the area of occupancy are not known, the geographic range of the clade is unlikely to reach the threshold for the clade to be listed as threatened ($<20,000 \text{ km}^2$, criterion D). Finally, too many variables are unknown for any quantitative analysis. If taken separately, an analysis of the extent of occurrence (EOO) and area of occupancy (AOO) for both A1 and A2 may result in the clades being listed as threatened.

Subclade A3&A4: southern subspecies of *D. cf. japonicus*

Similarly to A1&A2, the population size for this clade is not clearly defined, but the threats highlighted above might cause local declines. The population size is, however, likely to remain higher than the 10,000 individuals threshold (criterion C), and the decline is unlikely to be large enough ($\geq 30\%$; criterion A) for the subclade to be listed as threatened. Similarly, while the extent of occurrence and the area of occupancy are not known, the geographic range is unlikely to reach the threshold to be listed as threatened ($<20,000 \text{ km}^2$, criterion D). Too many variables are unknown for any quantitative analysis. We note that lineage A3 on Sado Island, if confirmed to be valid, may fit criteria A to be listed as threatened.

However, it remains to determine whether this mitochondrial lineage actually corresponds to a genuine taxon, as the Sado population is mainly composed of individuals featuring the widespread lineage A2.

Subclade B1&B2&B3: *D. j. japonicus*

As for the *D. cf. japonicus* subclades, the extent of occurrence and the area of occupancy are not known for *D. j. japonicus*, and the geographic range is unlikely to reach the threshold for the clade to be listed as threatened (<20,000 km², criterion D). Taken separately, mitochondrial lineages B1, B2, and B3 might be considered threatened based on criteria A, but their geographic boundaries are so far not determined.

Subclade B4&B5: *D. j. cf. stepheni*

Similarly to the other clades, the population size for this subclade is not clearly defined, but it is declining due to the threats highlighted above. The population size is, however, likely to be higher than 10,000 individuals (criterion C), and the decline is unlikely to be large enough ($\geq 30\%$; criterion A) for the clade to be listed as threatened. Similarly, while the extent of occurrence and the area of occupancy are not known, the geographic range of the clade is unlikely to reach the threshold for the clade to be listed as threatened (<20,000 km², criterion D). Finally, too many variables are unknown for any quantitative analysis. However, here the different lineages feature large distributions, so even if assessed separately, none would be listed as threatened based on range. Nevertheless, observed declines in the northern ranges, especially B5 populations in northern China, eastern Mongolia, and adjacent Russia, warrant further surveys and analyses.

Discussion

Although taxonomic inflation may be harmful to conservation (Zachos et al. 2013; Zachos 2015), species that are not described have a higher risk of extinction (Liu et al. 2022). In this study, we integrated phylogeographic and phenotypic analyses to delimit lineages in the *Dryophytes japonicus* complex as a first step to establish conservation baselines. Among the nine mitochondrial lineages previously documented (Dufresnes et al. 2016), we delimited two species, each with two clades that may be candidate subspecies pending future taxonomic efforts. To date, only half of the phylogeographic diversity has received taxonomic attention, and no lineage has been specifically assessed in a conservation framework.

Speciation in anuran amphibians is usually gradual, and genetic divergence can be used as a proxy for reproductive isolation (Dufresnes et al. 2021), thus contributing to integrative taxonomy (Padial et al. 2010). In an effort to apply newly developed species delimitation probabilities based on molecular divergence on Eastern

Palaearctic anurans, Dufresnes and Litvinchuk (2022) also recommended a species split between *D. japonicus* sensu stricto (clade B) and *D. cf. japonicus* (clade A). Namely, these two species diverged ~6 Mya based on mtDNA markers (Dufresnes et al. 2016), which usually translates into genomic incompatibilities consistent with advanced post-zygotic reproductive isolation that warrants species status (Dufresnes et al. 2021). Here, we provided new phylogenomic evidence for this deep divergence based on 42.8 kb nuclear loci, confirming the evolutionary independence of these clades. Accordingly, the divergence between the two species falls within the same time scale as other species-level Palaearctic hylids, e.g., newly-delimited species of the *Hyla annectans* complex (Wei et al. 2020; Yan et al. 2020) and in the *Hyla arborea* complex (Dufresnes et al. 2020a), where hybridisation only leads to geographically-restricted introgression.

The remaining diversity documented in the *D. japonicus* complex fits well with the current use of the subspecies rank within Eurasian hylids. First, the documented mitochondrial lineages (<3 Mya) generally match with the subspecies side of the grey zone of speciation, where genomes remain mostly compatible (Dufresnes et al. 2021). In spite of this rule of thumb, we note that experimental F1 crosses between *D. j. japonicus* and *D. j. stepheni* yielded offspring of lower fitness compared to intra-subclade crosses (Kawamura et al. 1990), despite their young evolutionary history (~1.2 My). As it goes against the speciation continuum of hylids, this result should be the focus of further investigations, and the taxonomy of the *D. japonicus* complex should be updated accordingly. Second, our phenotypic analyses reflected variation in call and morphology between subclades, thus warranting taxonomic recognition. Future comparisons between the several lineages found in each subclade might reveal additional taxa, e.g., between B4 and B5 (with the available names *stepheni* and *ussuriensis*, respectively; Nikolskii 1918), or even within the B5 haplogroup diversification. These lineages within the haplogroup B5 likely correspond to the same species, given that the level of genetic divergence matches intraspecific diversity in other hylids (e.g., *Dryophytes andersonii*; Warwick et al. 2021). However, since ghost mitochondrial lineages are widespread among anuran complexes (Dufresnes and Jablonski 2022), including *Hyla* (Dufresnes et al. 2019a), we do not recommend prematurely splitting *D. japonicus* and *D. cf. japonicus* solely based on closely related mtDNA variation.

In the *D. japonicus* complex, phenotypic analyses highlighted significant differences between and within species, although the latter pattern was not consistent between all call properties and morphometrics. In fact, phenotypic divergence did not follow molecular divergence, and, for instance, the deeply diverged and sea strait separated A1&A2 and B4&B5 (Fig. 1) remain phenotypically similar while belonging to distinct species. Such plesiomorphy or convergence may be related to their occurrence in similar landscapes and a tendency for characters to converge towards the same optimum (Arendt and

Reznick 2008) in the absence of pressure linked to sexual selection or isolation (Maan and Seehausen 2011). Interestingly, subclades with parapatric distributions, or that were parapatric in the recent past according to landscape modelling (Andersen et al. 2022), also featured significant differences in phenotypic characters, for instance, between the deeply-diverged A3&A4 vs. B1&B2&B3. These patterns could fit a hypothesis of reinforcement, where post-zygotic isolation triggers character shifts in traits involved in pre-mating isolation to avoid the costs of interbreeding (e.g., Talavera et al. 2021). Specific analysis of this contact zone in Central Japan will be necessary to test for reinforcement, especially since the genetic architecture of anuran reproductive isolation should theoretically not facilitate this hypothesis (Dufresnes and Crochet 2022). Here, our new barcoding data identified a putative transition near Kyoto, where mitotypes of both species coexist (namely B1 and A2, Fig. 1).

The absence of a clear-cut link between morphological, call, and genetic differences within the *D. japonicus* complex is not surprising. Phenotypic divergence (including mating calls, Forti et al. 2017) generally evolves slower than the establishment of reproductive isolation in anurans, so many young species/subspecies often remain cryptic in their early history, e.g., with little or no external diagnostic criteria (Dufresnes et al. 2021; Dufresnes and Crochet 2022). Interestingly, our morphometric analyses show that the PCs built from single traits were related to the characters influencing the shape of the vocal sacs (eyes and nostrils, and the head length; Borzée et al. 2013), which in turn may influence call variation. Yet, as species frequently hybridise in hylids (Dufresnes et al. 2015; Borzée et al. 2020a), it is also conceivable that recurrent hybridisation and genetic introgression contributed to homogenising emerging morphotypes. Accordingly, cryptic species are abundant among Eurasian hylids from the genera *Hyla* and *Dryophytes* (Dufresnes et al. 2020a; Wei et al. 2020).

None of the candidate species and subspecies we delimited is likely to be currently and immediately threatened, yet populations of all clades are expected to follow the global pattern of decline resulting from habitat loss and climate change (Blaustein et al. 1994; Green et al. 2020; Luedtke et al. 2023). In addition, due to their large geographic distribution, the widespread clades will probably reach assessment thresholds only after long-term declines. However, the narrow distribution of several mitochondrial lineages (e.g., A3, A4, B1, B2, and B3) calls to assess their evolutionary significance using genome-wide data and whether they correspond to genuinely unique populations and determine their EOO and AOO before they become extirpated. More positively, from a conservation perspective, some populations do not appear to be under threat, e.g., those of haplogroup B5b, ranging across the Democratic People's Republic of Korea, where the landscape is most adequate for amphibian species that tolerate agricultural wetlands, as observed through larger chorus sizes (Borzée et al. 2021b).

Data availability

The GenBank accession numbers for the new sequences of the four mitochondrial gene fragments are such as: 16S-ND1: [PQ788895–PQ789177](#). 12S: [PQ781706–PQ781924](#). COI: [PQ775660–PQ775874](#). *cytb*: [PQ781926–PQ782211](#).

Author contribution

Conceptualisation: AB, YJ, CD. Data curation: AB, SNO, JG, IVM, ZP, KY, TS, YY, NAS, DA, YB, IM. Formal analysis: AB, TS, YY, CD. Funding acquisition: AB, YJ. Investigation: AB. Methodology: AB, YJ, YY, CD. Project administration: AB, YJ, IVM, ZP, NAS, IM. Resources: AB, YJ. Software: AB, CD. Supervision: YJ, IVM. Validation: AB, YJ, TS, IM, CD. Visualisation: AB, SNO, JG, IVM, ZP, CD. Writing—original draft: AB, CD. Writing—review and editing: AB, YJ, SNO, JG, IVM, ZP, KY, TS, YY, NAS, DA, YB, IM, CD.

Acknowledgements

We are grateful to Sungsik Kong, Minjee Choe, Kyungmin Kim, Erdenetushig Purvee, Tumenkhuslen Munkhsaikhan, Solongo Gansukh, Jin-Long Ren, and Jia-Tang Li for their help in the field. This project was supported by the Research Fund for International Scientists (RFIS) from the National Natural Science Foundation of China (NSFC; W2432021) and the Foreign Youth Talent Program (QN2021014013L) from the Ministry of Science and Technology of the People's Republic of China awarded to AB, the Rural Development Agency of Korea (RS-2024-00397542) to YJ, and the Dobretsov Geological Institute of Siberian Branch of Russian Academy of Sciences (AAAA-A21-121011390004-6) to NAS. We are grateful to Günter Gollmann for the editorial support.

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Supplementary material 1

Details of the methods for genetic, acoustic, and morphometric analyses and a figure illustrating the results of the PCA of call properties

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Link: <https://doi.org/10.3897/herpetozoa.38.e137747.suppl1>